PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re the application of:

CARNEY

Serial No.: 10/628,391

Filed: July 29, 2003

For: INHIBITION OF NF-kB ACTIVATION

CLAIM TO PRIORITY

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

The benefit of the filing date of the prior foreign application filed in the following foreign country(ies) is hereby requested and the right of priority provided in 35 U.S.C. §119 is hereby claimed:

Ireland Application No. 2001/0069 filed 30 January 2001.

In support of this claim, filed herewith is a certified copy of said foreign application.

Respectfully submitted,

JACOBSON HOLMAN PLLC

By: _____
John C. Holman
Reg. No. 22,769

400 Seventh Street, N.W.
Washington, D.C. 20004-2201
Telephone: (202) 638-6666

Atty. Docket No.: P69048US0
Date: November 25, 2003
JCH:crj

Patents Office
Government Buildings
Hebron Road
Kilkenny

I HEREBY CERTIFY that annexed hereto is a true copy of the documents filed in connection with the following patent application:

Application No.	2001/0069
Date of Filing	30th January 2001
Applicant	The Provost Fellows and Scholars of the College of the Holy and Undivided Trinity of Queen Elizabeth, Near Dublin, a Registered charity of Ireland of College Green, Dublin 2, Ireland

Dated this 1st day of August 2003.

An officer authorised by the
Controller of Patents, Designs and Trademarks.

FORM NO. 1

REQUEST FOR THE GRANT OF A PATENT

PATENTS ACT, 1992

The Applicant(s) named herein hereby request(s)
__X__ the grant of a patent under Part II of the Act

_____ the grant of a short-term patent under Part III of the Act
on the basis of the information furnished hereunder.

1. Applicant(s)

Name: The Provost, Fellows and Scholars of the College of the Holy and Undivided Trinity of Queen Elizabeth, Near Dublin

Address: College Green
Dublin 2
Ireland

Description/Nationality

A Registered charity of Ireland

2. Title of Invention

"A Method"

3. Declaration of Priority on basis of previously filed application(s) for same invention (Sections 25 & 26)

Previous filing date	Country in or for which filed	Filing No.

4. Identification of Inventor(s)
Name(s) of person(s) believed
by Applicants(s) to be the inventor(s)

Name: Mohamed M Abdel-Latif, an Egyptian citizen of
Address: 71 New Ireland Road, Rialto, Dublin 8, Ireland

Name: Dermot Kelleher, an Irish citizen of
Address: 30 Royal Terrace West, Dun Laoghaire, Ireland

Name: Dermot O'Toole, an Irish citizen of
Address: 1 Place de l'Ecole, Paris 75001, France

Name: Henry J Windle, an Irish citizen of
Address: 15 Cherryfield Avenue Upper, Ranelagh Dublin 6, Ireland

5. Statement of right to be granted a patent (Section 17(2) (b)

The applicant derives the rights to the Invention by virtue of a Deed of Assignment dated December 17, 2000.

6. Items accompanying this Request – tick as appropriate

(i) __X__ prescribed filing fee (£100.00)

(ii) __X__ specification containing a description and claims

_____ specification containing a description only

__X__ Drawings referred to in description or claims

(iii) _____ An abstract

(iv) _____ Copy of previous application (s) whose priority is claimed

(v) _____ Translation of previous application whose priority is claimed

(vi) __X__ Authorisation of Agent (this may be given at 8 below if this Request is signed by the Applicant (s)

7. Divisional Application (s)

The following information is applicable to the present application which is made under Section 24 –

Earlier Application No: .....

Filing Date: .....

8. Agent

The following is authorised to act as agent in all proceedings connected with the obtaining of a patent to which this request relates and in relation to any patent granted -

Name	Address
John A. O'Brien & Associates	The address recorded for the time being in the Register of Patent Agents, and currently Third Floor, Duncairn House, 14 Carysfort Avenue, Blackrock, Co. Dublin, Ireland.

9. Address for Service (if different from that at 8)

As above

Signed _____ JOHN A. O'BRIEN & ASSOCIATES

Date January 30, 2001

" A METHOD"

Field of the Invention

This invention relates to a method for the prevention and reversal of NF-kappa B (NF-κB) activation in mammalian cells. In particular, the invention relates to the treatment of patients with chronic inflammatory diseases such as inflammatory bowel disease, rheumatoid/autoimmune arthritis, or any disease where the transcription factor NF-κB is transcriptionally active.

Expression of the transcription factor NF-κB is known to be increased in patients with inflammatory bowel disease, rheumatoid/autoimmune arthritis, asthma, septic shock, lung fibrosis, glomerulonephritis, atherosclerosis, autoimmune encephalomyelitis, and other chronic inflammatory disease states. In inflammatory bowel disease, for example, increased activation of NF-κB is thought to be involved in the regulation of the inflammatory response (1, 2). Inflammatory bowel disease (IBD) is a severe chronic inflammation treated mainly by immunosuppression (3). High levels of NF-κB activation have been shown in both Crohn's disease and animal models of inflammatory bowel disease. Crohn's disease is regarded as medically incurable. Treatment is aimed at inducing and maintaining remission and reducing complications. Crohn's disease is usually treated with 5-aminosalicylic acid, which has topical anti-inflammatory activity in the large and small bowel.

Novel inhibitors of NF-κB are currently under development for the treatment of inflammatory diseases such as asthma, rheumatoid/autoimmune arthritis and inflammatory bowel disease (4). Local administration of NF-κB p65 antisense phosphorothioate oligos in inflammatory bowel disease has been shown to abrogate the clinical and histological signs of colitis (5).

The majority of anti-inflammatory drugs fall into two categories, non steroidal anti inflammatory drugs (NSAIDs) and derivatives of the corticosteroids. Efficacy and toxicity vary. Regular users of NSAIDs are at serious risk of developing gastro intestinal disorders (9). NSAIDs work principally by interfering with the synthesis of inflammatory mediators (prostaglandins), whereas the corticosteroids have broad range effects due to their ability to regulate gene expression. Other classes of drugs are becoming available, for example leukotriene blockers (Singulair-Merck), which inhibit pro-inflammatory cell signalling mediated by this class of chemokines.

There is therefore an ongoing need for pharmaceuticals for the prophylaxis and/or treatment of diseases where the transcription factor NF-κB is transcriptionally active, especially chronic inflammatory disease.

Statements of Invention

According to the invention there is provided a *H. pylori* protein or derivative or fragment or mutant thereof capable of inhibiting the activation of NF-κB.

Preferably the protein is a thioredoxin or derivative or fragment or mutant thereof.

Most preferably the protein has the following amino acid sequence:

MSHYIELTEE NFESTIKKGV ALVDFWAPWC GPCKMLSPV
IDELASEYEG KAKICKVNTD EQEELSAKFG IRSIPTLLFT
KDGEVVHQLV GVQTKVALKE QLNKLLG

The invention also provides a thioredoxin or derivative or fragment or mutant thereof containing the redox active peptide sequence CGPC capable of inhibiting the activation of NF-κB.

The invention further provides prokaryotic or eukaryotic thioredoxins having potent immune-suppressive effects.

The invention also provides polypeptides containing the redox active peptide sequence CGPC, capable of inhibiting the activation of NF-κB.

The invention also provides a *H. pylori* protein having the following amino acid sequence:

MSHYIELTEE NFESTIKKGV ALVDFWAPWC GPCKMLSPV IDELASEYEG KAKICKVNTD EQEELSAKFG IRSIPTLLFT KDGEVVHQLV GVQTKVALKE QLNKLLG

The invention further provides use of a *H. pylori* thioredoxin protein or derivative or fragment thereof of the invention in a method for the prevention and/or treatment of inflammation, preferably for the prevention and/or treatment of inflammatory bowel disease.

The invention also provides use of a *H. pylori* thioredoxin protein or derivative or fragment thereof of the invention in a method for the prevention and/or treatment of rheumatoid /autoimmune arthritis, any chronic disease wherein NF-κB is transcriptionally activated, autoimmune arthritis or other autoimmune diseases, asthma, septic shock, lung fibrosis, glomerulonephritis, atherosclerosis and autoimmune encephalomyelitis.

The invention also provides use of a *H. pylori* thioredoxin protein or derivative or fragment or mutant thereof of the invention in blood transfusions and soft tissue injury.

The invention also provides a *H. pylori* thioredoxin protein or derivative or fragment or mutant thereof of the invention for use in the preparation of a medicament in the treatment and/or prophylaxis of any chronic disease wherein NF-κB is transcriptionally activated.

The invention further provides a protein of the invention for use in the preparation of a medicament for the treatment and/or prophylaxis of any chronic disease wherein NF-κB is transcriptionally activated.

Brief Description of the Drawings

The invention will more clearly understood from the following description thereof given by way of example only with reference to the accompanying drawings, in which:-

Fig. 1 is an electrophoretic mobility shift assay (EMSA) showing the effect of *H. pylori* thioredoxin on constitutive NF-κB activity in AGS cells (an adenocarcinoma cell line);

Fig. 2 is an electrophoretic mobility shift assay showing the time course of NF-κB inhibition upon treatment of AGS cells with *H. pylori* thioredoxin;

Fig. 3 is an electrophoretic mobility shift assay showing the effects of *H. pylori* thioredoxin on *H. pylori*-induced NF-κB activation in AGS cells; and

Fig. 4 is an electrophoretic mobility shift assay showing the effect of *H. pylori* thioredoxin on NF-κB activation by various stimuli.

Detailed Description

We have found a method for the prevention and reversal of NK-κB activation in mammalian cells by addition of effective inhibiting amounts of a *H. pylori* thioredoxin or a fragment or derivative or mutant thereof alone or in combination with a thioredoxin reductase regenerating system.

The invention is based on our finding that recombinant thioredoxin from the gastric pathogen *H. pylori* is a potent inhibitor of NF-κB activation *in vitro*. When added exogenously to AGS cells (an adenocarcinoma cell line) *in vitro* low doses of *H. pylori* thioredoxin (1-10 μg/ml; 70 nM-700 nM) inhibit constitutive NF-κB activity. In addition, *H. pylori* thioredoxin completely abrogates the pronounced NF-κB activity observed in AGS cells when NF-κB is activated by a variety of external stimuli including proinflammatory cytokines and phorbol esters. The ability of *H. pylori* thioredoxin to inhibit NF-κB activation *in vitro* suggest a potential therapeutic utility for thioredoxin as a novel approach for the treatment of patients with chronic inflammatory disease states such as autoimmunic arthritis, asthma, septic shock, lung fibrosis, glomerulonephritis, atherosclorosis, autoimmune encephalomyelitis, cystic fibrosis, rheumatoid arthritis, systemic inflammatory response syndrome and other NF-κB-mediated inflammatory disease states.

The present invention provides a protein, *H. pylori* thioredoxin, including a redox-active motif (CGPC), (cysteine-glycine-proline-cyseine), capable of inhibiting activation of the transcription factor NF-κB.

The polypeptide has the amino acid sequence:

MSHYIELTEE NFESTIKKGV ALVDFWAPWC GPCKMLSPV
IDELASEYEG KAKICKVNTD EQEELSAKFG IRSIPTLLFT
KDGEVVHQLV GVQTKVALKE QLNKLLG

SEQ ID NO 1

In the above sequence, individual amino acids are represented by the single letter code as follows:

Amino acid	Three letter abbreviation	One letter symbol
Alanine	Ala	A
Arginine	Arg	R
Asparagine	Asn	N
Aspartic acid	Asp	D
Asparagine or aspartic acid	Asx	B
Cysteine	Cys	C
Glutamine	Gln	Q
Glutamic acid	Glu	E
Glutamine or glutamic acid	Glx	Z
Glycine	Gly	G
Histidine	His	H
Isoleucine	Ile	I
Leucine	Leu	L
Lysine	Lys	K
Methionine	Met	M
Phenylalanine	Phe	F
Proline	Pro	P
Serine	Ser	S
Threonine	Thr	T
Tryptophan	Trp	W
Tyrosine	Tyr	Y
Valine	Val	V

The present invention also includes within its scope proteins derived from the native *H. pylori* thioredoxin identified above where such derivatives have redox-activity. These derivatives will normally be peptide fragments of the native protein which include the redox-active motif, but can also be functionally equivalent variants of the native thioredoxin modified by well known techniques such as site-directed mutagenesis. For example, it is possible by such techniques to substitute amino acids in a sequence with equivalent amino acids. Groups of amino acids known to be normally equivalent are:

(a)	A	S	T	P	G;
(b)	N	D	E	Q;	
(c)	H	R	K;		
(d)	M	L	I	V; and	
(e)	F	Y	W.		

Thioredoxin variants can be obtained by conventional gene engineering technology. For example, the amino acid sequence and base sequence of thioredoxin are known and described in numerous documents in the scientific literature. Based on the prior art documents, cDNA encoding natural thioredoxin can be obtained from an appropriate cDNA library. Then, a variant of the present invention can be obtained by, for example, site-directed mutagenesis (Nucleic Acid Research (1982) 10, 6487-6500).

The term derivative, fragment and mutant are understood to have the same meaning as commonly understood by one skilled in the art to which the invention belongs. A derivative is a chemical modification. A fragment may range in size from 4 amino acids to the entire amino acid sequence minus one residue. A mutant may have one or more changes in the molecular sequence of the gene.

The thioredoxin of the invention can be produced by isolation from *H. pylori*, using conventional purification techniques. However, it is recognised that for production of the protein in commercial quantities, production by synthetic routes is desirable. Such routes include the stepwise solid phase approach and production using recombinant DNA techniques. The latter route is preferred.

Stated generally, the production of thioredoxin by recombinant DNA techniques involves the transformation of a suitable host organism or cell with an expression vector including a DNA sequence coding for thioredoxin, followed by the culturing of the transformed host and subsequent recovering of the expressed thioredoxin. Such techniques are described generally in Sambrook et al. Molecular Cloning, 2nd edition, Cold Spring Harbour Press (1987).

The redox protein thioredoxin and the associated enzyme thioredoxin reductase (TR) constitute a thiol-dependent reduction-oxidation system that can catalyse the reduction of certain proteins by NADPH.

In its primary aspect, the present invention is directed to the provision of thioredoxin which is protective against inflammation. Subjects which are susceptible to inflammation are mammals including humans.

The concentration of thioredoxin which can be used ranges from about 1 μM to about 10 mM. The optimal concentration for intact reduced *H. pylori* thioredoxin appears to be a least 10 μM.

The thioredoxin compound can be administered orally to a patient in need of such treatment on a daily basis over an extended period of time. Alternatively the compound could be administered directly to the site of inflammation.

It should be recognised that the precise level of thioredoxin can be readily ascertained by a person skilled in the art in light of the present invention.

Thioredoxin and thioredoxin derived derivatives, fragments or mutants thereof may be administered directly, in the form of a formulation or any other pharmaceutically acceptable manner. Preferably such formulation includes an ingestable carrier which is a pharmaceutically acceptable carrier such as a capsule, tablet or powder. The formulation may also include an adjuvant or drug entity.

While the invention is broadly as defined above, it will be appreciated by those persons skilled in the art that it is not limited thereto but that it also includes embodiments of which the following description provides examples.

Materials and Methods used in the purification of thioredoxin from *H. pylori* and inhibition of NF-κB activity.

Materials 2',5'-ADP-agarose, Cibacron Blue 3GA, iminodiacetic acid-Sepharose 6B, ρ-aminobenzamidine-agarose, DTT (1,4-dithio-DL-threitol), *E. coli* thioredoxin and anti-*E. coli* thioredoxin were obtained from Sigma Chemical Co. Ltd., Poole, Dorset, U.K. Sephacryl S-300 was obtained from Pharmacia Biotech, Uppsala, Sweden. Isopropyl-β-D-thiogalactoside, NADPH, NADP+, and NADH were obtained from Boehringer Mannheim, Bell Lane, Lewes, East Sussex, UK. DEAE-52 was purchased from Whatman (Maidstone, UK). Factor Xa was purchased from New England Biolabs, Hertfordshire, U.K. All buffer reagents for SDS-PAGE were prepared in deionised water. The human gastric cancer cell line AGS and HuT 78, sezary lymphoma cells, were obtained from the European collection of Animal Cell Cultures (ECACC, Porton Down, Salisbury, UK). RPMI 1640 medium, fetal calf serum, penicillin, streptomycin, L-glutamine, Hank's Balanced salt solution (HBSS) and trypsin were obtained from

GIBCO BRL, life technologies Renfrewshire, Pasiley, Scotland. NF-κB consensus oligonucleotide was from Promega, poly(dI-dC) was from Pharmacia, Biosystems, Milton Keynes, UK. [γ^{32}P]ATP (35 pmol, 3000 Ci/mmol) was from Amersham International (Aylesbury, UK). Bovine albumin, ammonium persulphate, Nonidet P-40, PMA, IL-1β and PMSF were obtained from Sigma (Poole, Dorset, UK and St. Louis, MO., USA). All other chemicals were of analytical reagent grade.

Western blotting and SDS-PAGE. Discontinuous SDS-PAGE was performed essentially as described previously (11). Proteins from SDS-PAGE gels were electroblotted (0.9 mA/cm^2 for 1 h) to polyvinylidene difluoride membrane (Gelman) using a semi-dry blotting apparatus (LKB/Pharmacia), essentially as described by Towbin et al (12). Immunoblots were processed and developed by enhanced chemiluminesence as described previously (13). For N-terminal sequencing the protein was electroblotted to ProBlott and stained briefly with freshly prepared amido black.

Protein measurements. Protein was measured either by the method of Bradford or Markwell et al (14) with bovine serum albumin as the protein standard.

Bacterial strain and growth conditions. The reference strains of *H. pylori* used in this study (NCTC 11638 and 11637) were obtained from the National Collection of Type Cultures, Public Health Laboratory, London, U.K. All components for *H. pylori* culture media were obtained from Oxoid, Unipath Ltd., Basingstoke, Hampshire, U.K. *H. pylori* was grown under microaerobic conditions (Oxoid Campylobacter system, 5% O_2, 10% CO_2) for 4 days on 7% lysed horse blood Columbia agar at 37°C. Bacteria were harvested into RPMI medium without antibiotics and resuspended to yield a concentration of 6x10^8 organisms/ml and used immediately. *Escherichia coli* was used as a control.

Purification of thioredoxin reductase (TR). Agar-grown *H. pylori* was suspended in buffer A (20 mM Tris-HCl, pH 7.5) and subjected to sonication (4 x 1 min bursts) on ice using a Branson sonifier 450. After centrifugation to remove intact cells and cellular debris (12, 000 x g, 10 min, 4°C) the resulting supernatant was applied to a DEAE cellulose column (3.5 x 16 cm) equilibrated in buffer A. Thioredoxin reductase activity was eluted with a gradient (300 ml) of KCl (0 - 0.35 M) in buffer A. Active fractions were pooled, dialyzed against buffer B (50 mM Tris-HCl, pH 7.5) and applied to a Cibacron Blue 3GA column (1 x 3 cm). TR was eluted with a gradient of KCl (0 - 0.4 M). Active fractions were pooled, dialyzed against buffer B and applied to a small 2',5'-ADP agarose column (1 ml). Thioredoxin reductase was eluted upon application of 0.2 M KCl. The ion exchange and dye affinity chromatography steps were performed at room temperature and the ADP-Sepharose step was done at 4°C.

Gel filtration chromatography. A sonicate of *H. pylori* was prepared as described above and 0.5 ml (~10 mg protein/ml) of the material was applied to a column (diameter 1.5 cm; height 29.7 cm) of Sephacryl S-300 superfine (Pharmacia) equilibrated with phosphate buffered saline (pH 7.5) containing NaN_3 (0.02%, w/v). The protein was eluted with this same buffer (8.5 cm/h) and the collected fractions were assayed for both TR activity and total protein. The column was first calibrated with proteins of known molecular size (Pharmacia). Gel filtration over Sephadex G-50 (Pharmacia) was performed also in phosphate buffered saline (PBS).

Measurement of thioredoxin reductase activity. Thioredoxin reductase activity was assayed at 25°C in 0.1 M potassium phosphate buffer (pH 7.5) containing EDTA (1 mM), DTNB (5 mM) and NADPH (0.2 mM) in a final volume of 1.0 ml. The reaction was initiated by the addition of enzyme and the progress of the reaction was monitored by the increase in absorbance at 412 nm in a Pye Unicam 5625 spectrophotometer. One unit of enzyme activity is defined as the amount of

enzyme required to oxidize one μmol of NADPH per minute at 25°C, pH 7.5. Activity was calculated as μmol NADPH oxidized/min in accordance with the relationship ΔA412/(13.6 x 2). Thioredoxin reductase activity was assayed also using a minor modification of the insulin reduction assay (15). The reaction mixture consisted of 0.1 M potassium phosphate buffer (pH 7.0) containing EDTA (1 mM), insulin (0.1 mg/ml), NADPH (0.2 mM) and *H. pylori* histidine-tagged Trx (2 μM) in a final volume of 1 ml. The reaction was initiated by the addition the enzyme to the mixture at 25°C and the oxidation of NADPH was monitored at 340 nm. The amount of NADPH oxidized was determined from the relationship ΔA340/6.2.

Purification of native H. pylori Trx. Thioredoxin was purified by a combination of ion exchange chromatography on DEAE cellulose and gel filtration over Sephadex G-50. Fractions containing Trx were identified using the spectrophotometric insulin reduction assay (15).

Expression and purification of recombinant H. pylori Trx. Transformants of *E. coli* BL21(DE3)pLysS with plasmid pET-16b (Novagen) containing the Trx gene (HP 824) were grown at 37°C in LB broth supplemented with ampicillin (100 μg/ml) and chloramphenicol (30 μg/ml). *H. pylori* Trx was expressed as an N-terminal decahistidine fusion protein in *E. coli*. The gene coding for Trx was amplified by PCR using Expand™ (Boehringer Mannheim), using the amplification conditions recommended by the manufacturer. Under these conditions a single product was obtained and this was cloned into the expression plasmid via the *Bam*HI and *Nde*I restriction sites. The following primers were used: forward primer, 5'-CGCCATATGAGTCACTATATTGAATTAAC-3'; reverse primer 5'-CGCGGATCCGCCTAAGAGTTTGTTCAATTG-3'. Overexpression of the fusion protein was induced by adding 1 mM isopropyl-β-D-thiogalactoside at exponential phase and the incubation continued for 3 h at 37°C. The induced cells were harvested by centrifugation (10,000 x g, 15 min, 4°C), washed once

with 50 mM Tris·HCl (pH 7.5) and subjected to sonication (3 x 1 min). The soluble fusion protein was purified to homogeneity by metal chelate chromatography on a Ni^{2+} column (3 ml) according to the manufacturer's instructions. The protein was eluted with 0.4 M imidazole in 20 mM Tris·HCl (pH 7.5) containing 0.5 M NaCl. Typically, 2-3 mg of homogenous Trx/100 ml culture was obtained by this procedure. Both the histidine tagged fusion protein and the recombinant Trx obtained after cleavage of the histidine tail by Factor Xa were indistinguishable in their spectroscopic properties and redox behaviour.

Sequence analysis Multiple sequence alignments were made with the Clustal program. Amino-terminal sequence analysis of purified *H. pylori* Trx and TR was performed by Ms. Aine Healy at the National Food Biotechnology Centre, University College Cork using an Applied Biosystems automated sequencer.

Cell culture conditions AGS cells were grown in RPMI 1640 culture medium supplemented with 10% fetal calf serum, 100 units/ml penicillin, 100µg/ml streptomycin and 2 mM L-glutamine at 37°C (5% CO_2). For experiments, AGS cells were seeded at a density of $1x10^5$ cells/ml culture medium in 6-well plates and left overnight until confluent prior to experiments.

Coculture of AGS cells with H. pylori and other stimuli Confluent AGS cells were cocultured with or without *H. pylori, E.coli* ($6x10^8$ CFU/ml) or exposed to the cytokines interleukin-1beta (IL-1β)(10ng/ml) and tumor necrosis factor-alpha (TNF-α) (20ng/ml) or the mitogen phorbol 13-myristate 12-acetate (PMA) (20ng/ml) or incubated in acid media. To adjust media to the required pH value, 0.1 M HCl was added to the cell culture medium and titrated to the required pH. For controls, volumes of deionized water were added. Each experiment was performed in triplicate.

Preparation of nuclear extracts Nuclear extracts were prepared from unstimulated and stimulated AGS cells with various stimuli as described. Briefly, the cells were washed twice in ice-cold PBS, harvested by scraping with a cell scraper, and transferred into centrifuge tubes on ice. The cells were pelleted by centrifugation at 1400 rpm for 5 min and washed once in (1 ml) buffer A (10 mM HEPES (pH 7.9), 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM phenylmethylsulfonylfluride (PMSF) and 0.5 mM dithiothreitol (DTT) and centrifuged at 10,000 rpm for 10 min. The cell pellet was then resuspended in (20 µl) buffer A containing 0.1% Nonidet P-40 for 10 min. on ice and lysed cells were centrifuged at 10,000 rpm for 10 min. The supernatant was discarded and the nuclear pellet was extracted with (15 µl) buffer C (20 mM HEPES (pH 7.9), 420 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 25% glycerol and 0.5 mM PMSF) for 15 min. on ice. After incubation, the nuclei were centrifuged at 10000 rpm for 10 min. and the supernatant was diluted with 4 volumes of buffer D (10 mM HEPES (pH 7.9), 50 mM KCl, 0.2 mM EDTA, 25% glycerol and 0.5 mM PMSF). The nuclear extracts were used immediately or stored at -70°C until required. The protein concentration was determined on nuclear extracts by the method of Bradford.

Electrophoretic mobility-shift assays (EMSA) For binding assays, nuclear extracts (4 µg of protein) were incubated with 10000 cpm of the ^{32}P-labelled oligonucleotide (22 bp) containing the consensus sequence of the NF-κB (5`-AGT TGA GGG GAC TTT CCC AGG C-3`)(3`-TCA ACT CCC CTG AAA GGG TCC G-5`) that had been previously labelled with (γ-^{32}P)ATP at the 5'-ends with T4 polynucleotide kinase in 20 µl binding reaction in binding buffer (10 mM Tris, pH 7.5, 40% glycerol, 5 mM DTT, 1 mM EDTA, 100 mM NaCl and 0.1 mg/ml nuclease free bovine serum albumin) in the presence of 2 µg of poly(dI-dC) as non specific competitor. The reaction mixture was then incubated for 30 min at room temperature after the addition of the probe DNA. The binding reaction was terminated using a loading dye prior to adding the samples to the gels. The DNA-protein complexes were separated on 5% polyacrylamide gels (pre run at 80 V for

30 min) at 150 V for 1-2 h at room temperature. After electrophoresis was performed, the gels were dried and autoradiographed at -70°C for 24-36 h with intensifying screens.

Cell proliferation and toxicity assays AGS cells ($1x10^5$ cells/ml) were cultured in 96-well plates in triplicate overnight at 37°C. The cells were then incubated with or without thioredoxin for 24-72 h at 37°C. In some experiments, the cells were incubated with thioredoxin for 24 h at 37°C and then stimulated with either *H. pylori* or PMA for an additional 24 h at 37°C. To the cultured cells, 20 µl of freshly prepared PMS/MTS solution was added to each well and the plates were incubated for 4 h at 37°C. The absorbance of these wells was read at 490 nm using an ELISA plate reader. The average of the triplicate readings was taken for each sample. Under the experimental conditions and in the range of thioredoxin concentrations used, the cell viability was greater than 90%.

Flow cytometry analysis AGS cells were grown to confluence on 6-well plates and then the cells were incubated with thioredoxin for either 2 h at 37°C and then the cells were stimulated with *H. pylori* for 24 h at 37°C. The cells were washed with PBS and incubated for 30 min with antibodies to CD44 (L3D.1) and ICAM-1 at room temperature followed by washing and labelling with fluorescein isothiocynate (FITC)-conjugated rabbit F(ab)2' anti-mouse IgG (Dakopotts, Glostrup, Denmark). Samples were analyzed by flow cytometry in a FACscan (Becton Dickinson, Mountain View, CA).

The invention is not limited to the embodiments therein before described which may be varied in detail.

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CLAIMS

1. A *H. pylori* protein or derivative or fragment or mutant thereof capable of inhibiting the activation of NF-κB.

2. A *H. pylori* protein as claimed in claim 1 wherein the protein is a thioredoxin or derivative or fragment or mutant thereof.

3. A *H. pylori* protein as claimed in claim 1 or 2 wherein the protein has the following amino acid sequence:

MSHYIELTEE NFESTIKKGV ALVDFWAPWC GPCKMLSPV IDELASEYEG KAKICKVNTD EQEELSAKFG IRSIPTLLFT KDGEVVHQLV GVQTKVALKE QLNKLLG

4. A thioredoxin or derivative or fragment or mutant thereof containing the redox active peptide sequence CGPC capable of inhibiting the activation of NF-κB.

5. Prokaryotic or eukaryotic thioredoxins having potent immune-suppressive effects.

6. Polypeptides containing the redox active peptide sequence CGPC, capable of inhibiting the activation of NF-κB.

7. A *H. pylori* protein having the following amino acid sequence:

MSHYIELTEE NFESTIKKGV ALVDFWAPWC GPCKMLSPV IDELASEYEG KAKICKVNTD EQEELSAKFG IRSIPTLLFT KDGEVVHQLV GVQTKVALKE QLNKLLG

8. Use of a *H. pylori* thioredoxin protein or derivative or fragment thereof as claimed in any preceding claim in a method for the prevention and/or treatment of inflammation.

9. Use of a *H. pylori* thioredoxin protein or derivative or fragment thereof as claimed in claim 8 in a method for the prevention and/or treatment of inflammatory bowel disease.

10. Use of a *H. pylori* thioredoxin protein or derivative or fragment thereof as claimed in claim 8 in a method for the prevention and/or treatment of rheumatoid /autoimmune arthritis.

11. Use of a *H. pylori* thioredoxin protein or derivative or fragment thereof as claimed in claims 8 to 10 in a method for the prevention and/or treatment of any chronic disease wherein NF-κB is transcriptionally activated.

12 Use as claimed in claim 12 for the prevention and/or treatment of any of autoimmune arthritis or other autoimmune diseases, asthma, septic shock, lung fibrosis, glomerulonephritis, atherosclerosis and autoimmune encephalomyelitis.

13. Use of a *H. pylori* thioredoxin protein or derivative or fragment or mutant thereof as claimed in any preceding claim in soft tissue injury.

14. A *H. pylori* thioredoxin protein or derivative or fragment or mutant thereof as claimed in any of claims 1 to 7 for use in the preparation of a medicament in the treatment and/or prophylaxis of any chronic disease wherein NF-κB is transcriptionally activated.

15. A protein as claimed in any of claims 1 to 7 for use in the preparation of a medicament for the treatment and/or prophylaxis of any chronic disease wherein NF-κB is transcriptionally activated.

16. A *H. pylori* protein or derivative or fragment or mutant substantially as hereinbefore described.

17. Use of a *H. pylori* protein or derivative or fragment or mutant substantially as hereinbefore described

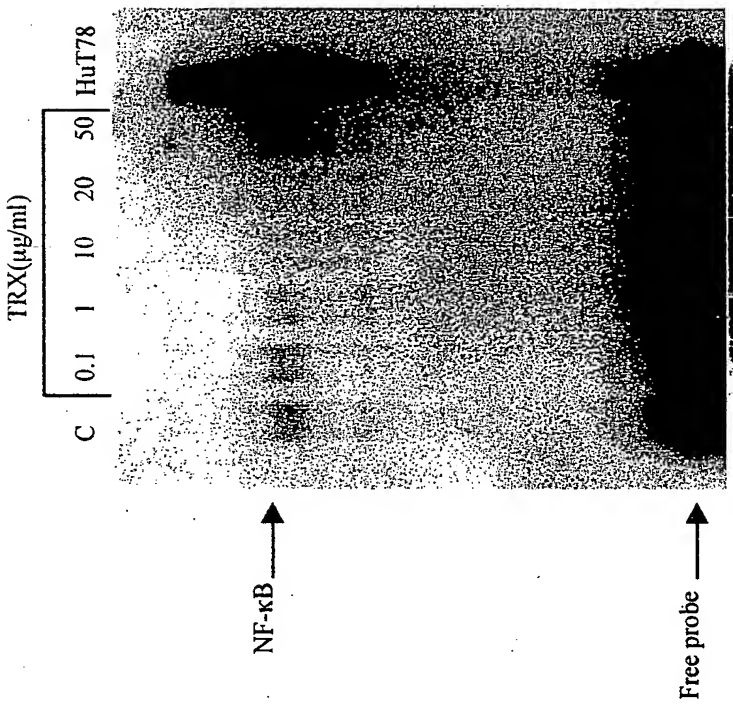

Figure 1. Effect of thiorcdoxin (TRX) on constitutive NFκB in AGS cells. AGS cells were treated with different doses of TRX (as indicated above each lane) for 2 h. and cell extracts were anaylzed in gel mobility-shift assay.

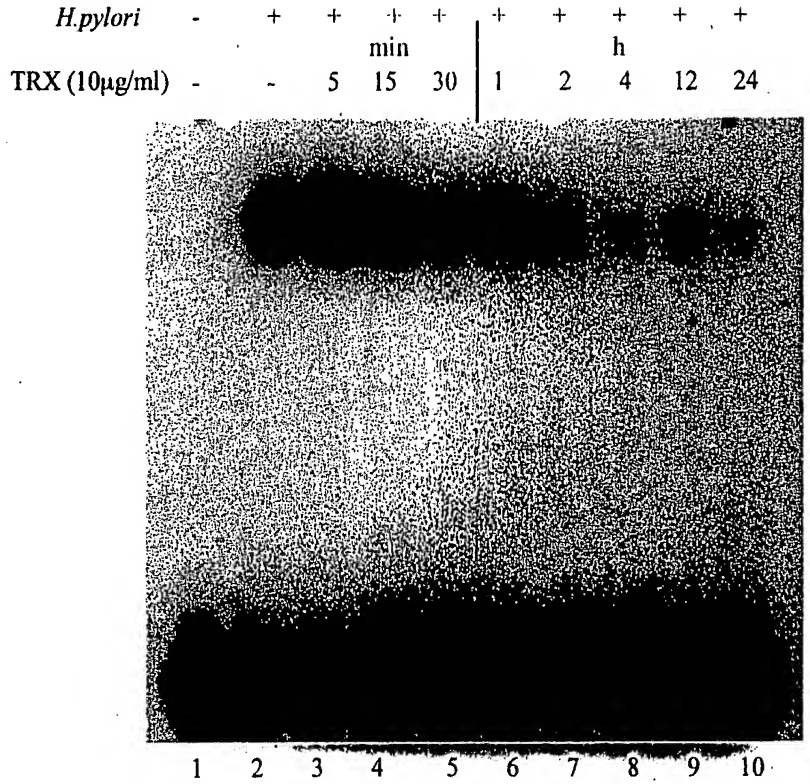

Figure 2. Time course of NFκB inhibition upon TRX treatment. AGS cells were treated with TRX (10 μg.ml) for different periods of time (as indicated above each lane) and then stimulated with *H. pylori* for 2 h and cell extracts were analyzed in EMSA

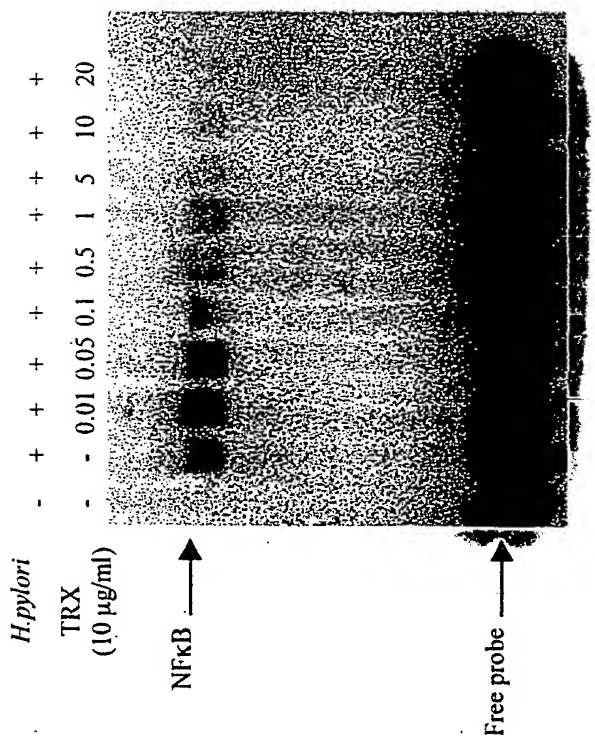

Figure 3. Effects of TRX on *H.pylori*-induced NFκB acitivation. AGS cells were treated with different doses of TRX (as indicated above each lane) for 2 h and then stimulated with *H. pylori* ($6x10^8$ cfu/ml) for 2 h and cell extracts were analyzed in EMSA.

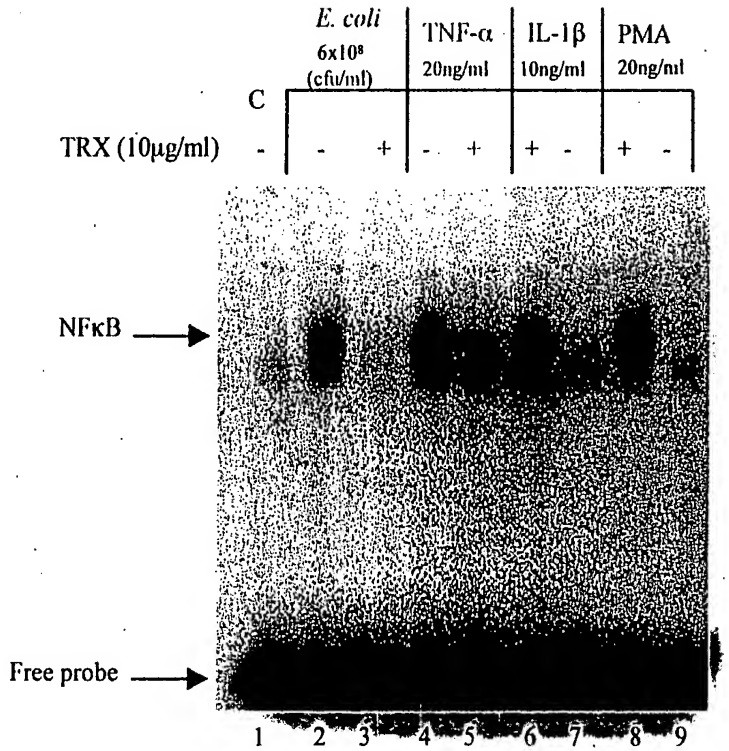

Figure 4 . Effects of thioredoxin (TRX) on NFκB activation by various stimuli. AGS cells were treated with TRX (10 μg/ml) for 2 h and then stimulated with either *E. coli* ($10x10^8$ cfu/ml), TNF-α (20ng/ml), IL-1β (10ng/ml) or PMA (20ng/ml) for 2 h and cell extracts were analyzed in EMSA.